



# A Coreceptor-Mimetic Peptide Enhances the Potency of V3-Glycan Antibodies

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**ABSTRACT** Broadly neutralizing antibodies (bNAbs) target five major epitopes on the HIV-1 envelope glycoprotein (Env). The most potent bNAbs have median half-maximal inhibitory concentration (IC<sub>50</sub>) values in the nanomolar range, and the broadest bNAbs neutralize up to 98% of HIV-1 strains. The engineered HIV-1 entry inhibitor eCD4-Ig has greater breadth than bNAbs and similar potency. eCD4-Ig is markedly more potent than CD4-Ig due to its C-terminal coreceptor-mimetic peptide. Here we investigated whether the coreceptor-mimetic peptide mim6 improved the potency of bNAbs with different epitopes. We observed that when mim6 was appended to the C terminus of the heavy chains of bNAbs, this sulfopeptide improved the potency of all classes of bNAbs against HIV-1 isolates that are sensitive to neutralization by the sulfopeptide alone. However, mim6 did not significantly enhance neutralization of other isolates when appended to most classes of bNAbs, with one exception. Specifically, mim6 improved the potency of bNAbs of the V3-glycan class, including PGT121, PGT122, PGT128, and 10-1074, by an average of 2-fold for all HIV-1 isolates assayed. Despite this difference, 10-1074 does not induce exposure of the coreceptor-binding site, and addition of mim6 to 10-1074 did not promote shedding of the gp120 subunit of Env. Mixtures of 10-1074 and an Fc domain fused to mim6 neutralized less efficiently than a 10-1074/mim6 fusion, indicating that mim6 enhances the avidity of this fusion. Our data show that mim6 can consistently improve the potency of V3-glycan antibodies and suggest that these antibodies bind in an orientation that facilitates mim6 association with Env.

**IMPORTANCE** HIV-1 requires both the cellular receptor CD4 and a tyrosine-sulfated coreceptor to infect its target cells. CD4-Ig is a fusion of the HIV-1-binding domains of CD4 with an antibody Fc domain. Previous studies have demonstrated that the potency of CD4-Ig is markedly increased by appending a coreceptor-mimetic sulfopeptide to its C terminus. We investigated whether this coreceptor-mimetic peptide improves the potency of broadly neutralizing antibodies (bNAbs) targeting five major epitopes on the HIV-1 envelope glycoprotein (Env). We observed that inclusion of the sulfopeptide dramatically improved the potency of all bNAb classes against isolates with more-open Env structures, typically those that utilize the coreceptor CXCR4. In contrast, the sulfopeptide improved only V3-glycan antibodies when neutralizing primary isolates, on average by 2-fold. These studies improve the potency of one class of bNAbs, show that coreceptor-mimetic sulfopeptides enhance neutralization through distinct mechanisms, and provide insight for the design of novel multispecific entry inhibitors.

**KEYWORDS** CCR5, V3-glycan, antibody, envelope glycoprotein, human immunodeficiency virus, tyrosine sulfation

The HIV-1 envelope glycoprotein (Env) is a trimer of heterodimers, consisting of three gp120 surface proteins and three gp41 transmembrane proteins (1–4). Viral entry initiates when gp120 binds its host cell receptor CD4. This association induces confor-

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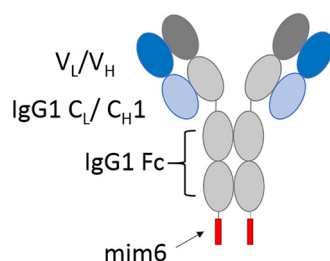
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mational changes in Env, exposing the coreceptor-binding site and facilitating its association with an HIV-1 coreceptor, principally CCR5 or CXCR4 (5–8). Coreceptor association promotes additional conformational changes in gp41 that lead to association of the gp41 fusion peptide with the target cell membrane, mixing of virion and cell lipids, fusion pore expansion, and ultimately entry of the viral capsid into the cytoplasm (5, 6).

Env is the only viral protein on the surface of an HIV-1 virion and therefore is the sole target for neutralizing antibodies. Partly as a consequence, Env is heterogeneous and highly glycosylated, and its diversity among circulating HIV-1 isolates is very high (9–14). Most antibodies elicited by infection are poorly neutralizing or have a limited breadth, recognizing only a small fraction of HIV-1 strains (15–18). However, 10 to 30% of HIV-1-infected patients develop broadly neutralizing antibodies (bNAbs) against HIV-1 (19–21). There are to date five major classes of bNAbs, covering most of the HIV-1 Env ectodomain surface. These five classes include bNAbs targeting the CD4-binding site (CDbs antibodies), bNAbs that specifically recognize glycosylations at the V3 loop (V3-glycan antibodies), bNAbs that target the V2-glycan apex of the Env trimer (apex antibodies), bNAbs that recognize the gp120/gp41 interface, and bNAbs recognizing the membrane-proximal external region of gp41 (MPER antibodies) (22, 23). In addition to bNAbs, additional classes of antibodies have been described, such as V3-loop antibodies (distinct from V3-glycan antibodies) and antibodies whose epitope is exposed by CD4 binding (CD4-induced [CD4i] antibodies). These latter antibodies are more common in infected individuals, but they do not neutralize primary isolates because their epitopes are occluded in the closed conformation of the Env trimer (12, 15–18). The broadest bNAbs can neutralize up to 98% of HIV-1 isolates, and the most potent have half-maximal inhibitory concentrations ( $IC_{50}$ s) in the nanogram/milliliter range (24–27). However, epitopes of these bNAbs also contact residues that are not highly conserved, and mutation of these residues facilitates viral escape without affecting the viral fitness (28–30). Indeed, many studies have shown that treatment with a single bNAbs will result in rapid viral escape (31–35).

The N terminus of CCR5 includes multiple sulfated tyrosines, a property it shares with the other described HIV-1 coreceptors, including CXCR4. The sulfotyrosines of CCR5 have been shown to be important for binding to a conserved coreceptor-binding region of Env located in the C4 region, in the bridging sheet, and at the base of the V3 loop of gp120 (36–38). Some CD4i antibodies, such as E51 and 412d, which bind this region, also have sulfotyrosines in their heavy-chain complementarity-determining regions 3 (CDR-H3) (39, 40). Sulfopeptides derived from this CDR-H3 region, named CCR5mim1 and CCR5mim2 (mim1 and mim2), are able to neutralize some HIV-1 isolates when fused to an Fc domain of IgG1 (41, 42). These mim1-Ig- and mim2-Ig-sensitive isolates include most lab-adapted strains and isolates obtained late in an untreated infection. The Envs of these isolates more frequently occupy an open conformation, presumed to be similar to the CD4-bound state of most primary isolates (40, 43). When either of these sulfopeptides are fused to the C terminus of CD4-Ig, the potency of this inhibitor increases by around 10-fold (44). Unlike CD4-Ig, the resulting construct, eCD4-Ig, does not promote infection of CCR5-positive cells when CD4 is limiting, presumably because the sulfopeptide prevents direct association between Env and the coreceptor (44).

Here we investigated whether the coreceptor-mimetic peptide mim6 (45) fused to the C termini of bNAbs of various classes could improve their neutralization as it does for CD4-Ig. We observed that mim6 consistently improved the potency of all V3-glycan antibodies tested, whereas it improved the potency of all other bNAbs classes only for Envs that could be neutralized by the peptide alone. We excluded several reasons why V3-glycan antibodies, but not antibodies of other classes, could be improved by mim6. For example, we did not detect any conformational changes induced by the V3-glycan antibody 10-1074, and no significant shedding of gp120 from Env-expressing cells was observed with this antibody, whether or not it was fused to mim6. We conclude that



**FIG 1** Fusion of the coreceptor-mimetic sulfopeptide mim6 to bNAbs. The coreceptor-mimetic peptide mim6 was fused to the C termini of the heavy chains of bNAbs. Gray represents the heavy chain, with the variable region in dark gray. The light chain is represented in blue, while the darker blue corresponds to the variable region. mim6 is represented in red.

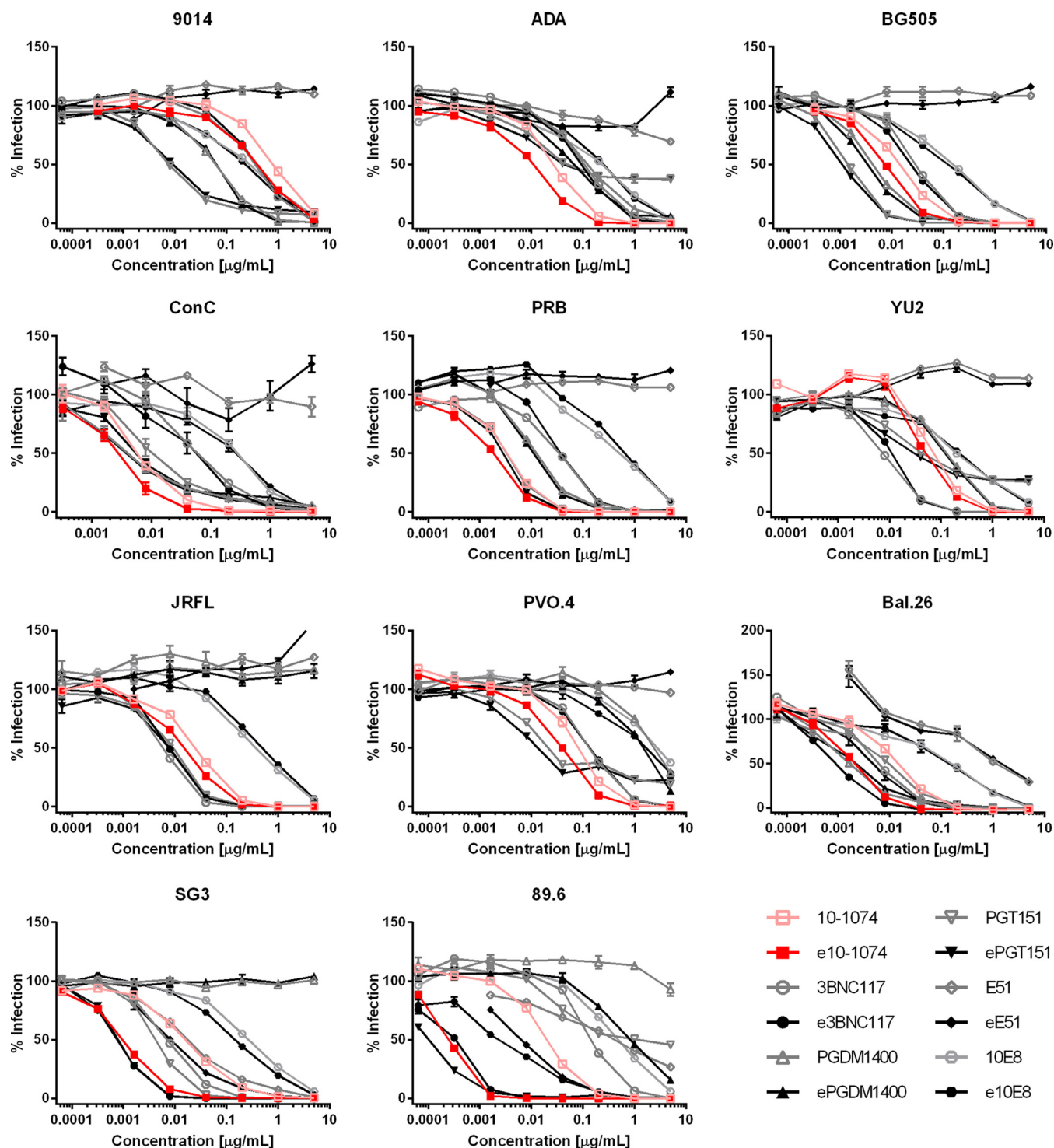
mim6 can improve the potency of one class of neutralizing antibodies, perhaps because these antibodies bind Env in an orientation favorable to simultaneous binding of mim6.

## RESULTS

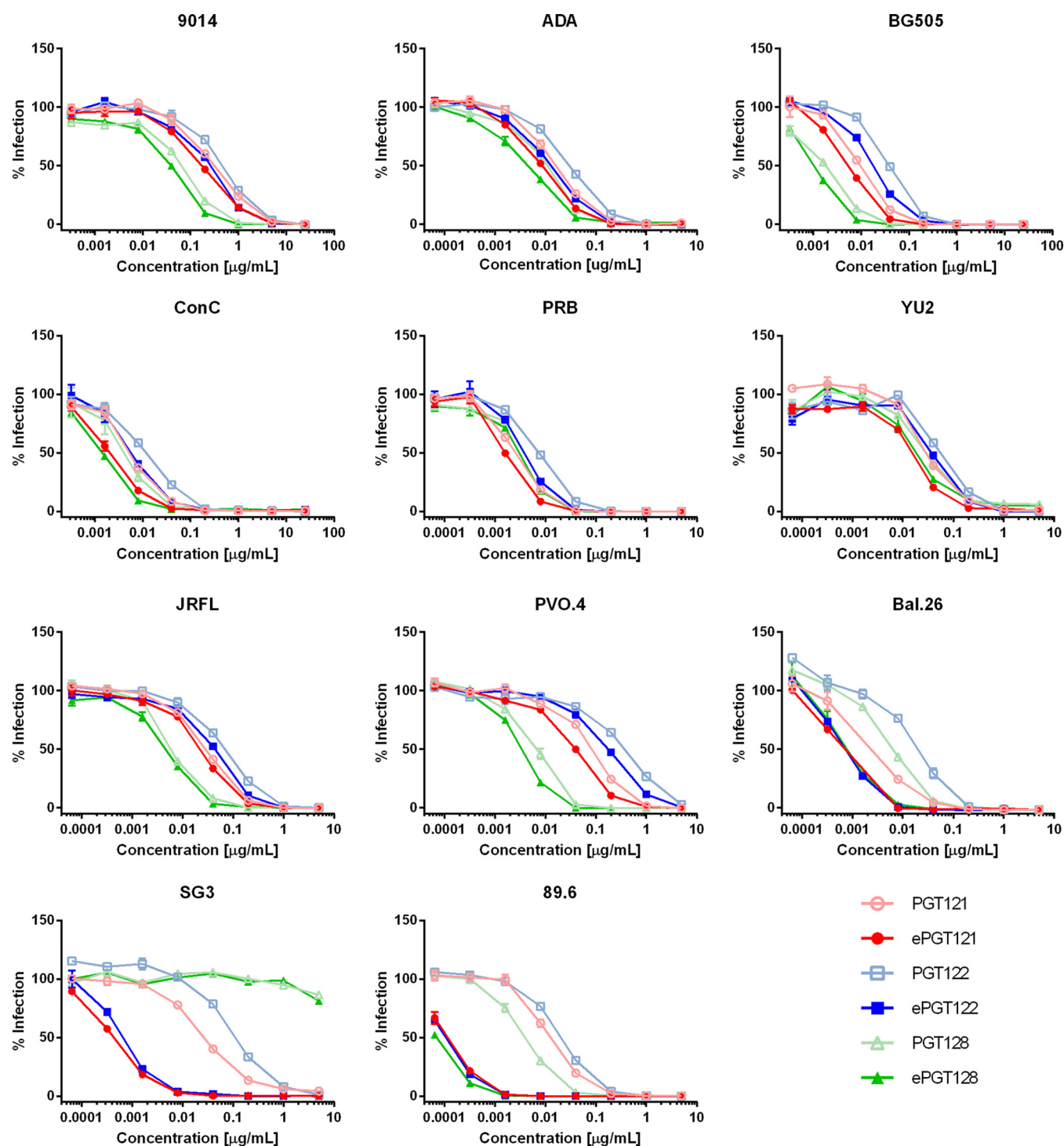
**mim6 improves potency of all classes of bNAbs only for sulfopeptide-sensitive strains.** When a coreceptor-mimetic sulfopeptide like mim6 is fused to CD4-Ig at its C terminus, the neutralization potency of CD4-Ig is increased by approximately 10-fold (44). We investigated whether mim6 could improve the potency of bNAbs from different classes. To do so, we fused mim6 to the C terminus of one bNAb of each major class (Fig. 1), namely, 3BNC117 (CD4bs), 10-1074 (V3-glycan), PGDM1400 (V2-apex), PGT151 (gp120-gp41 interface), 10E8 (MPER), and E51 (CD4i). To distinguish antibodies fused to mim6 from the parental form, we appended an “e” to the name (e.g., e10-1074). We then compared the neutralization efficiencies of bNAbs with and without the mim6 fusion using a TZM-bl neutralization assay (Fig. 2). We observed that mim6 improved the potency of all bNAbs for HIV-1 isolates that were sensitive to neutralization by the sulfopeptide, namely, the 89.6 and SG3 isolates (41, 42). Additionally, mim6 improved the ability of 10-1074 to neutralize all 11 HIV-1 strains tested by at least 2-fold. This improvement was markedly greater for sulfopeptide-sensitive isolates, up to 58-fold for 89.6. The ability of mim6 to enhance 10-1074-mediated neutralization of all isolates contrasts with the case for antibodies of every other bNAb class.

**mim6 improves V3-glycan antibodies for all HIV-1 strains tested.** To determine if these differences in neutralization are specific to 10-1074 alone or are a common property of all V3-glycan bNAbs, we fused mim6 to the V3-glycan bNAbs PGT121, PGT122, and PGT128. As with 10-1074, mim6 improved the neutralization efficiency of all three bNAbs for every isolate assayed (Fig. 3). Most HIV-1 isolates tested were neutralized around 2-fold more effectively in the presence of the peptide. Again, inclusion of mim6 markedly improved neutralization of sulfopeptide-sensitive isolates, up to 153-fold in the case of the bNAb PGT122 and the isolate 89.6. Thus, mim6 improved the potency of every V3-glycan antibody tested against every isolate, suggesting that the mechanism of improvement by mim6 is common to the entire V3-glycan class. Figure 4 summarizes the  $IC_{50}$ s for all antibodies tested, for all isolates, excluding those sensitive to the sulfopeptide alone (89.6 and SG3). As indicated, the mim6 sulfopeptide significantly improved neutralization of all four V3-glycan antibodies (Fig. 4A and B) but did not improve neutralization mediated by bNAbs from any other class (Fig. 4A).

**e10-1074 is more potent than a mixture of 10-1074 and an Fc-mim6 fusion.** We sought to determine if the fusion of mim6 to 10-1074 was necessary for the increase in potency observed with e10-1074. We therefore compared the neutralization efficiencies of e10-1074 to those of a mixture of 10-1074 and Fc-mim6, a fusion in which mim6 was fused to the C terminus of a human IgG1 Fc domain (Fig. 5). In these experiments, both 10-1074 and Fc-mim6 were provided at the indicated concentrations so that the same numbers of 10-1074 variable chains and mim6 peptides were compared. We observed again that e10-1074 was more potent than 10-1074. However, the addition of Fc-mim6



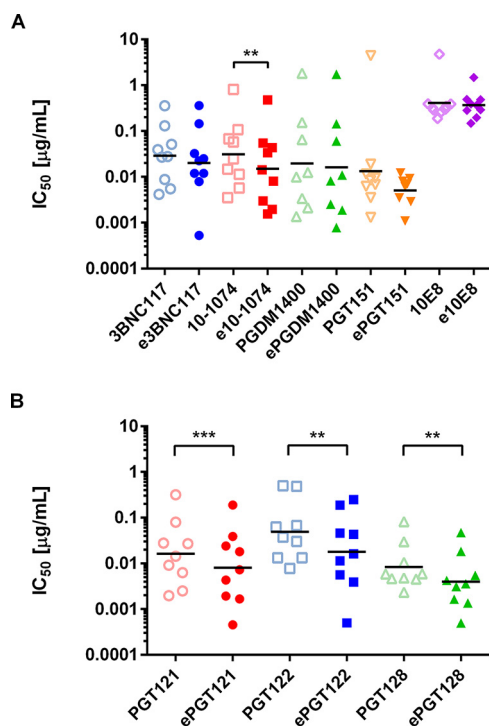
**FIG 2** mim6 improves the potency of 10-1074 against primary isolates and of all classes of bNAbs against R5X4 and X4 isolates. Neutralization efficiencies of representatives of different classes of bNAbs with and without mim6 ("e" indicates the mim6 attachment) were measured using a TZM-bl neutralization assay. Antibodies were preincubated for 1 h with HIV-1 pseudovirus expressing the indicated Env proteins. At 48 to 72 h after addition of TZM-bl cells, luciferase expression was measured and normalized to expression in the absence of any inhibitor. SG3 and 89.6 are X4 and R5X4 Envs, respectively, with an "open" conformation and are sensitive to neutralization by sulfopeptide-Fc constructs (41, 42). The remainder of the Envs are derived from R5 isolates. Note that except for the V3-glycan bNAb 10-1074, the sulfopeptide did not improve neutralization of these latter Envs. Error bars indicate standard errors of the means (SEM) of triplicates.



**FIG 3** mim6 improves the potency of three additional V3-glycan bNAbs. The V3-glycan antibodies PGT121, PGT122, and PGT128 were compared to the same antibodies fused to mim6. As for Fig. 2, neutralization efficiencies were determined using a TZM-bl neutralization assay, and error bars indicate SEM of triplicates.

to 10-1074 did not improve neutralization of any of the five isolates assayed. As expected, Fc-mim6 alone did not neutralize any isolate except 89.6, as previously reported (41). These data suggest that the 10-1074 variable chains of e10-1074 help to localize the mim6 sulfopeptide to the coreceptor-binding site.

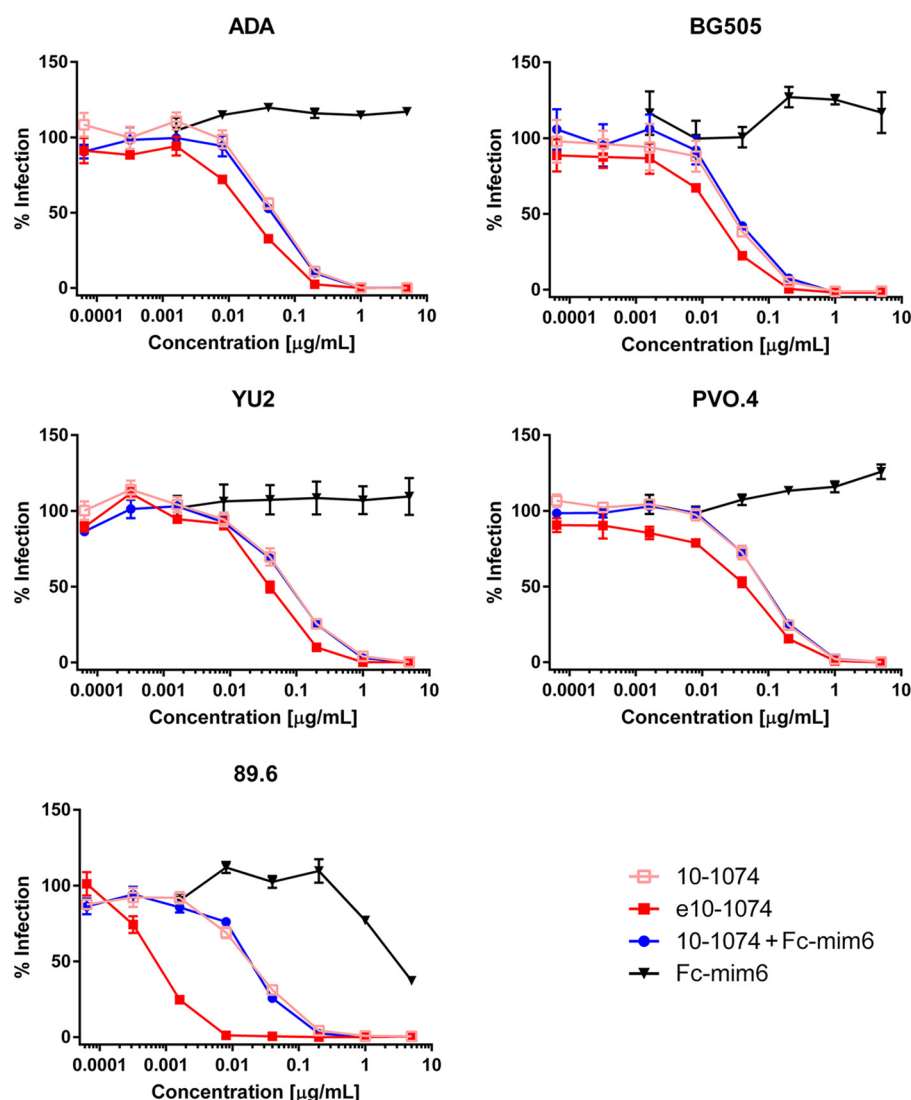
**Binding of e10-1074 saturates at lower concentrations than binding of 10-1074 to open and closed Envs.** To further explore how mim6 improves the potency of



**FIG 4**  $IC_{50}$  values of bNAbs. The  $IC_{50}$  values from Fig. 2 (A) and 3 (B) were plotted for all primary isolates, with the “open” HIV-1 isolates SG3 and 89.6 excluded. Geometric means for neutralized isolates are indicated with horizontal lines. mim6 significantly improved the potency of the V3-glycan antibodies 10-1074 ( $P = 0.002$ ), PGT121 ( $P = 0.0009$ ), PGT122 ( $P = 0.009$ ), and PGT128 ( $P = 0.008$ ) but not that of any of the other bNAbs tested (ratio paired Student's  $t$  test).

V3-glycan antibodies, we investigated the ability of 10-1074 and e10-1074 to bind cell surface-expressed Env. We tested 89.6 $\Delta$ CT, a sulfopeptide-sensitive (“open”) isolate, and BG505 $\Delta$ CT, a sulfopeptide-resistant (“closed”) Env with truncations of the gp41 cytoplasmic tails. This “ $\Delta$ CT” truncation prevents internalization of Env and allows for high levels of expression on the cell surface. HEK293T cells transfected to express these Envs were incubated with increasing concentrations of 10-1074 and e10-1074. At low concentrations the binding of 10-1074 and that of e10-1074 were similar, but binding of e10-1074 to both Envs saturated at lower concentrations than for 10-1074 (Fig. 6). These data suggest that 10-1074 and e10-1074 bind these Envs with similar affinities but that additional 10-1074 epitopes are occluded by the bound sulfopeptide. We have previously reported that the sulfopeptide of eCD4-Ig similarly results in lower occupancy of Env at saturation (44).

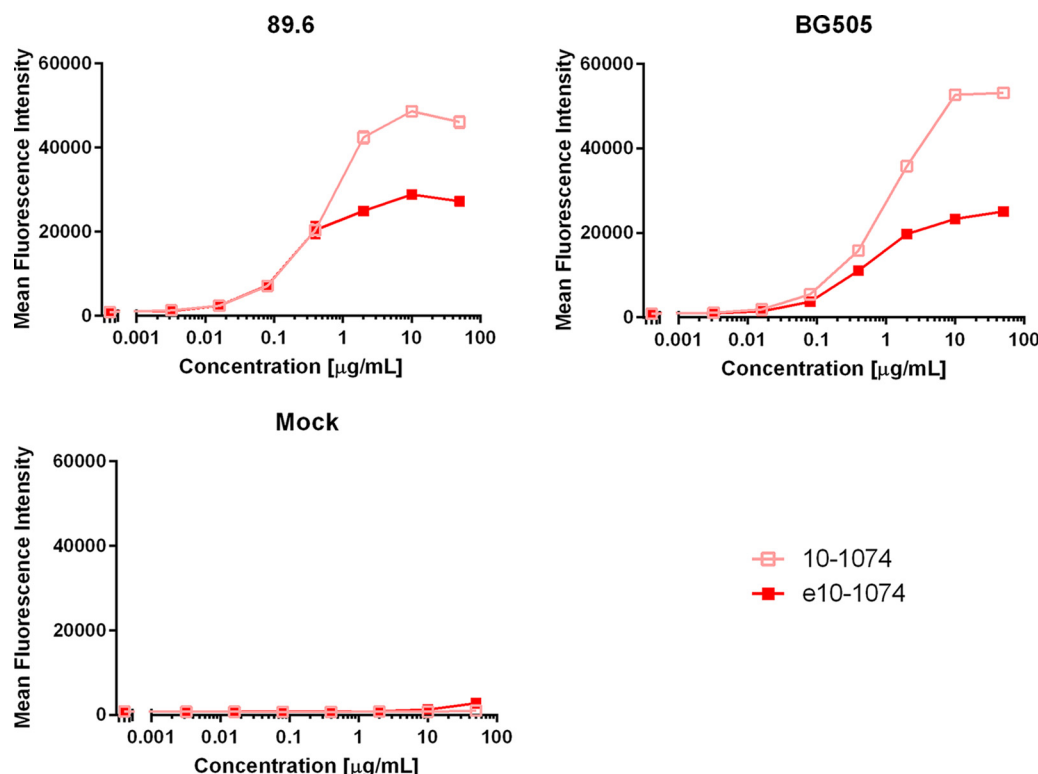
**10-1074 binding does not induce conformational changes as receptor binding does.** Binding of CD4-Ig to Env induces conformational changes, exposing the coreceptor-binding site, which for most isolates is conformationally masked and inaccessible in the absence of CD4 (5, 12). One possible reason that mim6 improves the neutralization of V3-glycan antibodies in particular is that this class of antibodies also might partially expose the coreceptor-binding region. To assess this possibility, we transfected HEK293T cells with BG505 $\Delta$ CT and incubated them with CD4-mIg, 10-1074-mIg, or 3BNC117-mIg, with mIg indicating that the Fc domains of these antibodies were of murine origin. CD4-mIg served as a positive control, and the CD4bs antibody 3BNC117-mIg served as a negative control (46). We then incubated these cells with mim2-Ig (which is different from mim6 at its penultimate glycine) and 17b, a coreceptor-binding site antibody. We have previously shown that mim2-Ig can, like 17b, recognize the CD4-bound state of Env (41, 42, 47). As expected, CD4-mIg improved binding of 17b and mim2-Ig to the BG505 $\Delta$ CT trimer. However, neither 3BNC117-mIg nor 10-1074-mIg improved binding of these coreceptor-binding site ligands (Fig. 7).



**FIG 5** Mixtures of 10-1074 and Fc-mim6 neutralize less efficiently than e10-1074. The neutralization efficiencies of 10-1074, e10-1074, Fc-mim6, and mixtures of 10-1074 and Fc-mim6, each present at the concentration of e10-1074, were analyzed using a TZM-bl neutralization assay. Antibodies were preincubated for 1 h with HIV-1 pseudovirus expressing the indicated Env proteins. TZM-bl cells were then added, and 72 h later, luciferase expression was measured and normalized to expression in the absence of any inhibitor. Note that the mixture of 10-1074 and Fc-mim6 neutralizes similarly to 10-1074 alone. Only 89.6 is sensitive to neutralization by Fc-mim6 alone, as previously reported (41, 42). Error bars indicate standard errors of means (SEM) of triplicates.

These data exclude the possibility that 10-1074 can itself expose the coreceptor-binding site of Env in the manner of CD4-Ig.

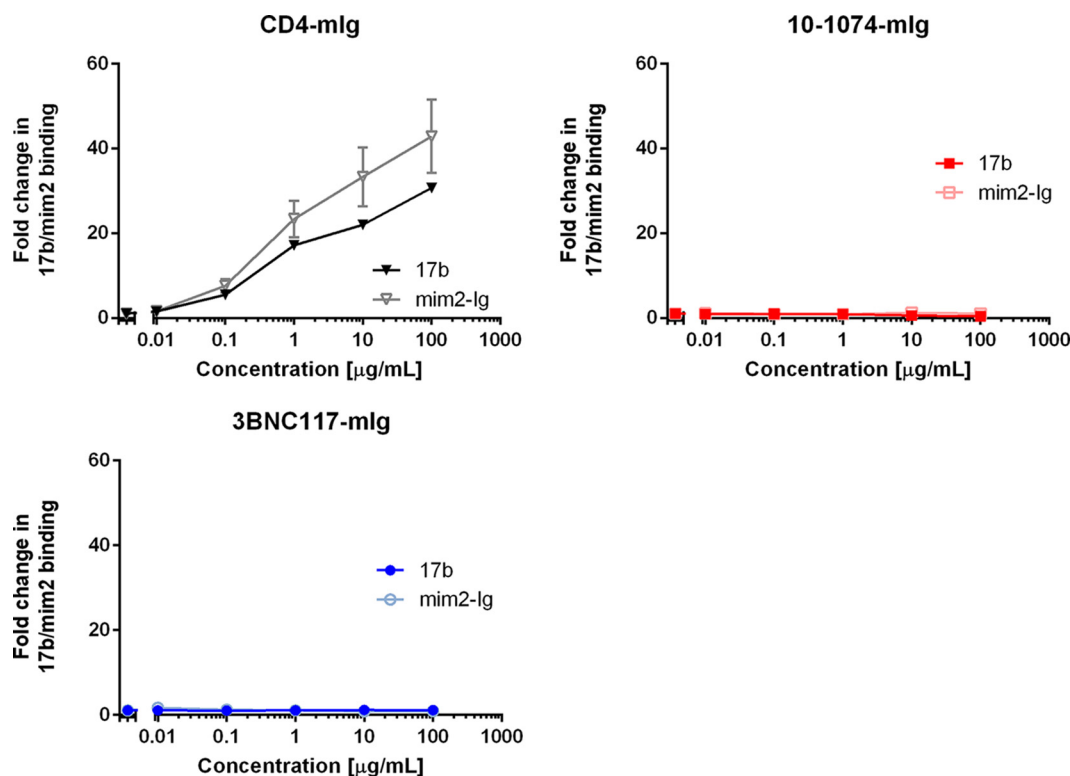
**e10-1074 does not induce more shedding than 10-1074.** Previous studies have shown that incubation of cell-expressed Env trimers with soluble CD4 (sCD4) or CD4-Ig promoted dissociation of gp120 from gp41, a phenomenon described as “shedding” (48). We have previously shown that eCD4-Ig promotes more efficient shedding than CD4-Ig (45). We therefore investigated whether e10-1074 promoted greater shedding than 10-1074. To do so, HEK293T cells were transiently transfected to express full-length Env trimers of two HIV-1 isolates (89.6 and YU2) and incubated for one hour with 10-1074, e10-1074, or eCD4-Ig as a positive control. As previously reported, eCD4-Ig promoted concentration-dependent shedding of gp120 into the supernatant of Env-expressing cells, whereas no shedding was observed with 10-1074 or e10-1074, even with Envs that were sulfopeptide sensitive (Fig. 8). We conclude that mim6 does not



**FIG 6** Binding of e10-1074 to Env saturates at lower concentrations than that of 10-1074. HEK293T cells were transfected to express the Envs of 89.6 ("open") or BG505 ("closed"), both with truncated cytoplasmic tails. The transfected cells were incubated with the indicated concentrations of 10-1074 or e10-1074 for 1 h on ice, and bound protein was detected by flow cytometry. Error bars indicate SEM of duplicates. Data are representative of three independent experiments with similar results.

increase the ability of 10-1074 to promote gp120 shedding and therefore that shedding did not account for the greater neutralization potency of e10-1074 relative to 10-1074.

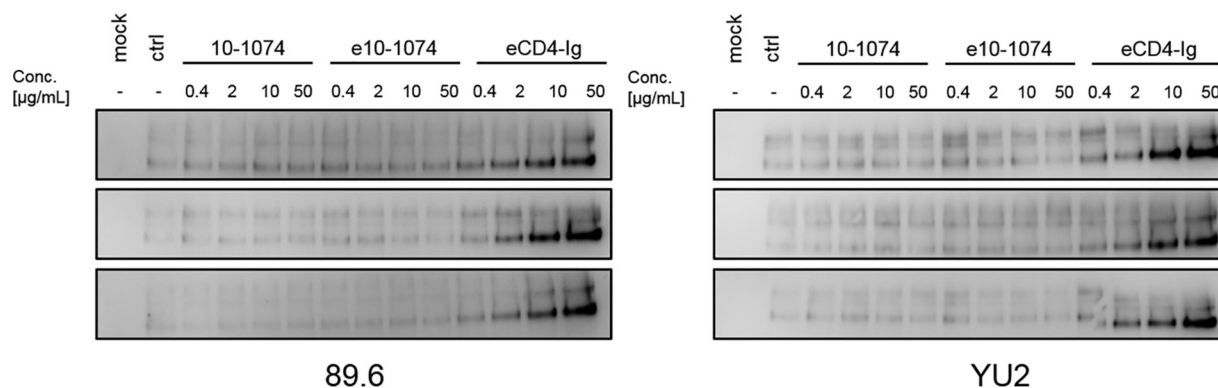
**mim6 increases ADCC activity of 10-1074, 3BNC117, and PGT128 against 89.6-infected cells.** Antibody-dependent cell-mediated cytotoxicity (ADCC) can eliminate HIV-1-infected cells and may be useful in strategies to reduce the size of the reservoir of latently infected cells (49). eCD4-Ig has been shown to mediate more potent ADCC activity than CD4-Ig, with an efficiency roughly proportional to its greater efficiency of neutralization (44, 50). We compared the abilities of 10-1074 and e10-1074 to mediate ADCC (Fig. 9). 10-1074 and e10-1074 both mediated efficient killing of infected cells. However, both inhibitors mediated killing of cells expressing YU2 Env with similar efficiencies. In contrast, e10-1074 mediated killing of cells expressing the sulfopeptide-sensitive 89.6 Env with markedly greater efficiency than 10-1074. This difference in ADCC activity correlates with the large difference with which these inhibitors neutralize the 89.6 isolate and is again consistent with the suggestion that e10-1074 interacts differently with open, sulfopeptide-sensitive Envs (e.g., 89.6) than with sulfopeptide-resistant Envs (e.g., YU2) (see also neutralization with Fc-mim6 in Fig. 5). To see whether the difference in ADCC activity was common for all classes of bNAbs or just for the V3-glycan class, we also tested the ADCC activity of 3BNC117 (CD4bs) and PGT128 (V3-glycan) with and without peptide. Again, mim6 did not improve the ADCC activity against cells infected with the YU2 isolate, while the ADCC activity for cells infected with 89.6 was markedly improved in the presence of the peptide. The inability of mim6 to improve ADCC activity against YU2 may reflect the inability of this ADCC assay to detect modest differences in activity, or it may suggest that the lower occupancy by e10-1074 observed in Fig. 5 impairs cell killing.



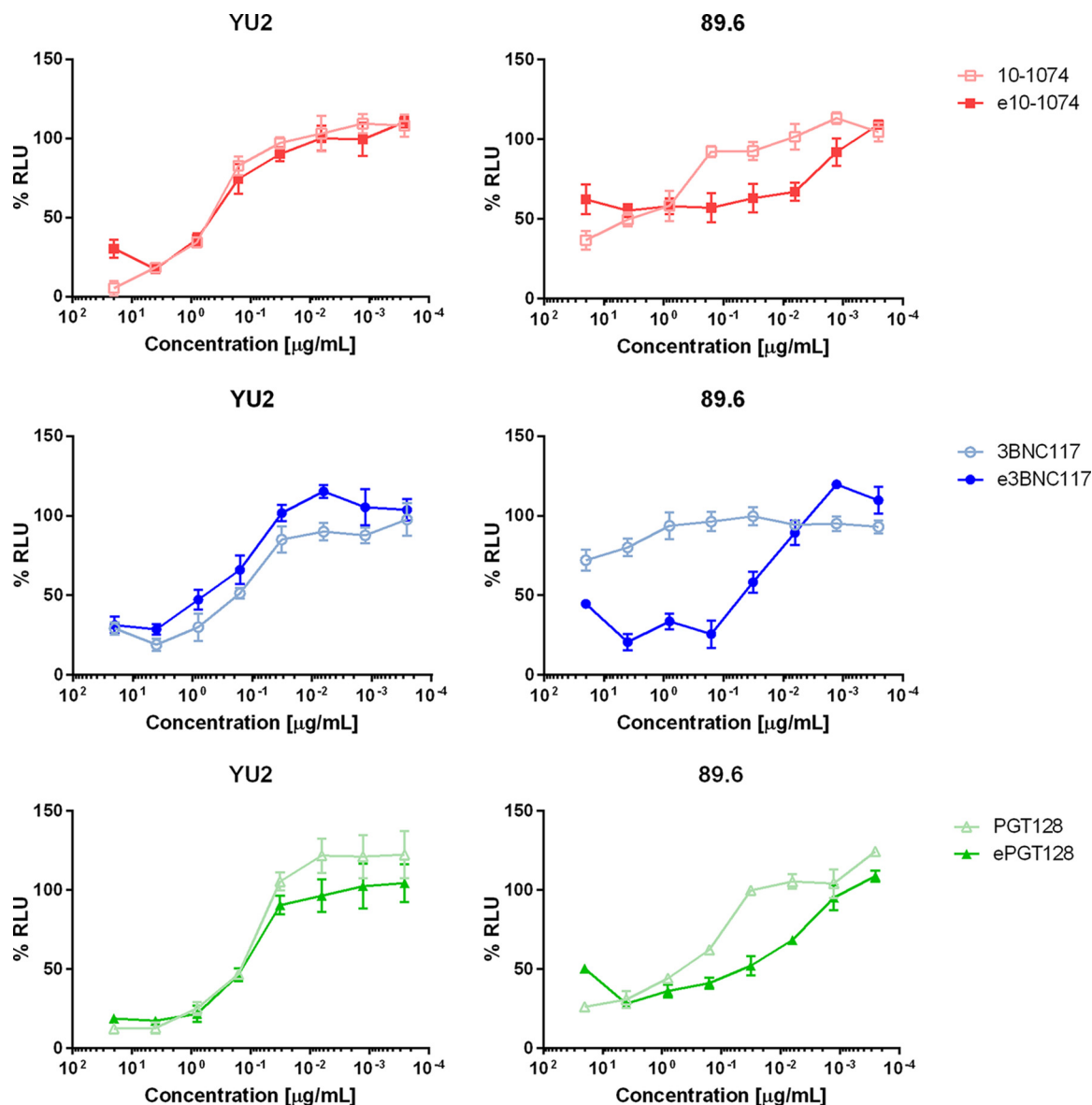
**FIG 7** 10-1074 and 3BNC117 do not expose the coreceptor-binding site of Env. HEK293T cells were transfected to express the BG505 Env with a truncated cytoplasmic tail and preincubated with CD4-mIg, 10-1074-mIg, or 3BNC117-mIg for 1 h. mIg indicates that the Fc domain is murine IgG2a. Cells were then incubated with mim2-Ig or 17b, and mim2-Ig and 17b binding was measured by flow cytometry using an APC-coupled anti-human Fc antibody. Note that, as expected, CD4-mIg induces 17b and mim2-Ig binding to Env. However, 10-1074-mIg and 3BNC117-mIg did not affect binding of these coreceptor-binding-site ligands. Error bars indicate SEM of duplicates. Data are representative of three independent experiments.

## DISCUSSION

The amino termini of CCR5 and other G-protein-coupled receptors that can function as HIV-1 coreceptors include several sulfated tyrosines (36–38). Antibodies that recognize the coreceptor-binding site of Env similarly contain sulfated tyrosines in their CDR-H3 regions (39, 51, 52). The CDR-H3 of one such antibody, E51, is the origin of the coreceptor-mimetic peptides used here, including mim6 (41, 42). Each of these peptides has a pattern of sulfotyrosines similar to that of CCR5 and presumably bind Env



**FIG 8** e10-1074 does not induce shedding of Env. HEK293T cells were transiently transfected to express the Envs of 89.6 or YU2. At 48 h after transfection, cells were incubated for 1 h with increasing concentrations of 10-1074, e10-1074, or eCD4-Ig. Supernatants were separated by SDS-PAGE, and the gels were analyzed by Western blotting with sheep anti-gp120 polyclonal serum. Mock indicates untransfected cells. Ctrl indicates cells transfected with Env without inhibitor. Results from three independent experiments are shown.



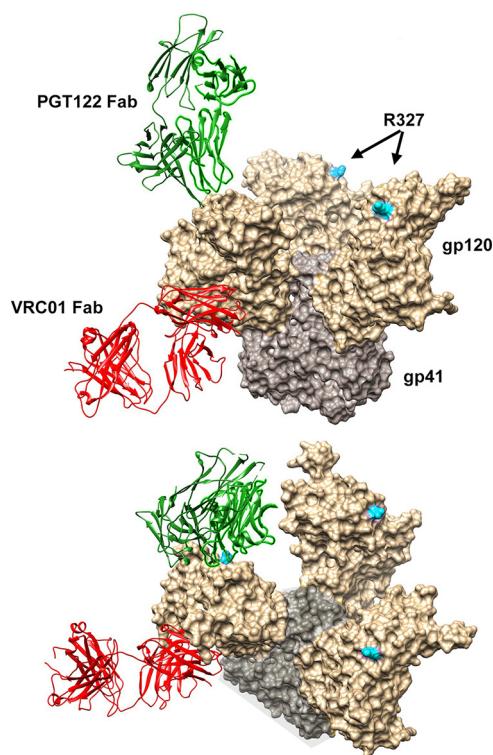
**FIG 9** ADCC activities of e10-1074, ePGT128, and e3BNC117. To determine ADCC activities, CEM.NKR-CCR5-LTR-Luc cells were infected with HIV-1 isolate YU2 or 89.6. At 3 to 4 days postinfection, a 10:1 ratio of effector to target cells was added in the presence of the indicated concentrations of 10-1074, e10-1074, 3BNC117, e3BNC117, PGT128, or ePGT128. After 8 h, ADCC activities were measured as a loss of luciferase signal in relative light units (RLU). Error bars indicate SEM of triplicates. The graphs represent two independent experiments with similar results.

in the same way as the CCR5 N terminus (39, 51, 52). While these coreceptor-mimetic peptides alone have low affinities and can neutralize only a small number of HIV-1 isolates, fusion of these peptides to CD4-Ig has been shown to markedly increase the potency of CD4-Ig (41, 42, 44). These constructs also prevent enhancement of infection in the absence of cellular CD4, one of the major problems with the use of CD4-Ig as a therapeutic (44, 53). Here we assessed the impact of the coreceptor-mimetic sulfopeptide mim6 when it was fused to the C termini of various antibodies. We observed that mim6 improved neutralization for all classes of antibodies if the isolate was already sensitive to neutralization by the sulfopeptide alone, suggesting that the Envs of these isolates adopt a more open conformation in which the coreceptor-binding site is exposed. Inclusion of the sulfopeptide resulted in dramatic improvements of neutralization potency for these isolates, up to 270-fold. However, the sulfopeptide did not significantly improve the potency against most isolates when fused to antibodies of

most classes, indicating that, unlike CD4, these antibodies do not promote a more open Env conformation. In contrast, fusion of mim6 with antibodies of the V3-glycan class showed consistent improvement in potency against sulfopeptide-resistant Envs tested, albeit by a modest 2-fold. This observation was consistent for all four members of this bNAb class tested, namely, 10-1074, PGT121, PGT122, and PGT128, suggesting that this effect applies to all antibodies of this class.

To evaluate why mim6 enhanced the potency of V3-glycan antibodies, but not antibodies of any other class, against sulfopeptide-resistant viruses, we explored several hypotheses. First, we speculated that V3-glycan antibodies might help to expose the coreceptor-binding site similarly to CD4-Ig. However, 10-1074 did not enhance binding of mim2-Ig or the CD4i antibody 17b, indicating that this bNAb did not increase coreceptor-binding site exposure. We also explored whether mim6 increased the ability of 10-1074 to promote the irreversible inactivation of Env as indicated by gp120 shedding, but we observed no difference between 10-1074 and e10-1074 in this property. We observed, however, that although 10-1074 and e10-1074 bound cell-expressed Env with similar efficiencies, binding of e10-1074 to Env-expressing cells saturated with less antibody bound. This observation suggests that e10-1074 binds its epitope at the base of the V3 loop of one protomer of the Env trimer. The sulfopeptide can then associate with Env in such a way that the 10-1074 epitope on an adjacent protomer is occluded. Consistent with such a model, mixtures of 10-1074 and Fc-mim6 neutralized with an efficiency similar to that of 10-1074 alone and less than that of e10-1074. Thus, e10-1074 appears to bind Env with higher avidity than 10-1074. This model is also supported by the structure of Env in complex with various V3-glycan bNAbs, which suggests that the sulfopeptide of e10-1074 could associate with the coreceptor-binding site of an adjacent protomer and that the Fc domain would then block access of another V3-glycan antibody to that protomer (2, 54–58). However, we cannot fully exclude the possibility that cross-linking of distinct Envs on the virion surface also contributes to the greater potency of e10-1074.

This explanation leaves several questions unanswered. First, how does the mim6-binding epitope, presumably the coreceptor binding site, become accessible to the sulfopeptide of e10-1074? We have shown that, unlike CD4-Ig, 10-1074 cannot expose the coreceptor-binding site. However, Envs are presumed to “breathe”; that is, they sample multiple conformations over time (59–62). It is possible that binding of e10-1074 to its V3-glycan epitope positions the sulfopeptide in such a way that it can bind the coreceptor-binding site when this site is transiently exposed (Fig. 10). Second, why does mim6 enhance the potency of V3-glycan bNAbs against all isolates but fails to do so with antibodies of other classes? The previous explanation suggests that only V3-glycan antibodies assume an orientation that allows mim6 to bind the coreceptor-binding site. Inspection of structures in which various antibodies are complexed to Env suggests that CD4bs (Fig. 10), gp120/gp41 interface, and MPER antibodies would position the sulfopeptide away from the coreceptor-binding region (27, 63–66). A notable exception to this rule is the V2-glycan/apex antibodies, but unlike other classes of antibodies, this class locks Env in a closed conformation that would preclude exposure of the coreceptor-binding site (67, 68). Finally, why does mim6 markedly improve the potency of all antibodies against isolates whose coreceptor sites are already exposed? Here the answer appears to be straightforward: antibodies bearing mim6 can neutralize isolates with open Envs in at least two independent ways, namely, by direct interaction with the coreceptor binding site and through recognition of the original epitope of the bNAb. However, it is not clear why mim6 increases neutralization of these open Envs so dramatically, by more than 100-fold in several cases. This observation is more puzzling because the sulfopeptide neutralizes even sulfopeptide-sensitive isolates with potencies in the 1 to 10  $\mu$ M range, compared to antibodies that neutralize at 1 to 10 nM. It is possible that sulfopeptide interactions have rapid on-rates, thereby localizing the antibody to the Env trimer and facilitating binding of these antibodies to their higher-affinity epitope. Although these hypotheses need to be confirmed experimentally, our current data make clear that mim6 fusions with V3-



**FIG 10** The orientation of V3-glycan bNabs may facilitate simultaneous binding of mim6 to the sulfopeptide-binding region. Two views of the structure of a partially open HIV-1 Env trimer (B41 isolate; PDB 5VN8) bound to the CD4-binding site bNAb VRC01 (red) or the V3-glycan bNAb PGT122 (green) are shown. Env subunits gp120 and gp41 are shown in tan and gray, respectively. Arginine 327, at the base of the gp120 third variable loop and marking a key sulfotyrosine-binding pocket (42), is indicated in cyan. The structure was generated by overlaying PDB 5VN8 with PDB 4LST (VRC01 bound to the ZM176.66 gp120 protein) and PDB 4NCO (PGT122 bound to the Env of BG505). Note that only the V3-glycan bNAb PGT122 may be oriented to allow the Fc-sulfopeptide domain to simultaneously bind the sulfopeptide-binding region. Specifically, the C terminus of the C1 domain of PGT122 is 105 Å from the  $\alpha$ -carbon of the nearest accessible gp120 arginine 327, whereas the C terminus of the VRC01 C1 domain is 142 Å from its nearest accessible arginine 327. Note that an antibody Fc domain extends more than 80 Å and with its 15-amino-acid hinge region can extend beyond 105 Å.

glycan antibodies function differently than such fusions with either CD4-Ig or bNabs of other classes. While no bNab-sulfopeptide fusion is likely to be clinically useful at this point, analysis of the properties of these fusions may inform the development of other multispecific HIV-1 entry inhibitors.

## MATERIALS AND METHODS

**Cells and plasmids.** TZM-bl cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (contributed by John C. Kappes, Xiaoyun Wu, and Tranzyme Inc.). TZM-bl cells were generated from JC.53 cells, a cell line stably expressing CD4, CCR5, and CXCR4. Additionally, copies of the luciferase and  $\beta$ -galactosidase genes under the control of the HIV-1 promoter were introduced (69–73). TZM-bl cells and human embryonic kidney cells (HEK293T) cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) (Corning) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin-streptomycin (Gibco), and 10 mM HEPES (Corning) at 37°C with 5% CO<sub>2</sub> content. Expi293 cells (Thermo Fisher Scientific) were grown in Expi293 expression medium (Thermo Fisher Scientific) at 37°C and 8% CO<sub>2</sub> content at 125 rpm in a New Brunswick S41i shaker incubator (Eppendorf). CEM.NKR-CCR5-LTR-Luc ADCC target cells with a Tat-inducible luciferase gene (a generous gift from Michael Alpert, Emme Inc.) were generated from CEM.NKR-CCR5 CD4<sup>+</sup> T cells obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (contributed by Alexandra Trkola) (74). CEM.NKR-CCR5-LTR-Luc target cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 25 mM HEPES, 2 mM L-glutamine (Gibco), and 0.1 mg/ml Primocin (InvivoGen) at 37°C with 5% CO<sub>2</sub> content. KHYG-1 natural killer (NK) cells expressing human CD16 were grown in RPMI 1640 medium supplemented with 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml Primocin, 1  $\mu$ g/ml cyclosporine, and interleukin-2 at 37°C with 5% CO<sub>2</sub> content (75, 76).

The variable heavy and light chains of 3BNC117, 10-1074, PGT121, PGT122, PGT128, PGDM1400, PGT151, 10E8, and E51 were cloned into human IgG1 expression vectors. 3BNC117 and 10-1074 IgG1

expression vectors were provided by Michel Nussenzweig. 10E8 expression vector was obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (contributed by Mark Connors) (27). PGT121, PGT122, and PGT128 were provided by Dennis Burton. mim6 with a four-glycine linker was synthesized as a gblock (IDT) and was attached to the C terminus of the heavy chain through restriction enzyme digest. Plasmids expressing CD4-Ig and eCD4-Ig versions have been previously described (44). Antibodies with murine Fc domains were generated by replacing genes encoding the human IgG1 Fc domain with those for murine IgG2a. HIV-1 gp160 expression vectors, vectors expressing Env with a cytoplasmic tail truncation ( $\Delta$ CT), and vectors expressing pNL4.3 $\Delta$ env were previously described (7, 42, 52, 77, 78).

**Protein production and purification.** Expi293 cells were grown to a density of  $3 \times 10^6$  cells/ml in 100 ml Expi293 expression medium. For antibody production, cells were transfected with 23  $\mu$ g heavy chain and 23  $\mu$ g light chain using ExpiFectamine (Thermo Fisher Scientific) according to the manufacturer's instructions. For antibodies with mim6, 11.2  $\mu$ g of TPST2 was cotransfected to ensure sufficient sulfation of mim6. For CD4-Ig production, Expi293 cells were transfected with 45  $\mu$ g of CD4-Ig, and for eCD4-Ig production, cells were transfected with 45  $\mu$ g eCD4-Ig and 11.2  $\mu$ g TPST2. At 5 days posttransfection, medium was collected, centrifuged at 2,500 rpm for 10 min at 4°C, and filtered with a 0.45- $\mu$ m rapid-flow filter (Thermo Fisher Scientific). Protein was purified with MabSelect SuRe columns (GE Healthcare) and eluted with IgG elution buffer (Thermo Fisher Scientific) into 1 M Tris-HCl buffer, pH 9 (G Biosciences). Buffer was exchanged twice with phosphate-buffered saline (PBS) in Amicon Ultra-15 centrifugal filter units (Millipore) at  $4,000 \times g$  and 4°C and was concentrated to 1 mg/ml. The protein concentration was measured at a wavelength of 280 nm with a NanoDrop (Thermo Fisher Scientific). Protein was stored at 4°C.

**Pseudovirus production.** HEK293T cells were transiently transfected with 45  $\mu$ g of a plasmid expressing Env of the indicated HIV-1 isolate and 45  $\mu$ g of pNL4.3 $\Delta$ Env, an HIV-1 expression vector lacking a functional *env* gene. Medium was changed at 12 h posttransfection. At 48 h after the medium change, supernatants were collected and filtered through a 0.45- $\mu$ m syringe filter. Aliquoted pseudoviruses were stored at  $-80^\circ\text{C}$ .

**TZM-bl neutralization assay.** TZM-bl neutralization assays have been previously described (41, 44, 46, 79). In brief, HIV-1 pseudoviruses were incubated with titrated amounts of the indicated antibodies in DMEM with 10% FBS for 1 h at 37°C. TZM-bl cells were detached using trypsin and diluted to 100,000 cells/ml. Cells were then added to the pseudovirus-inhibitor mix and incubated for 48 to 72 h at 37°C. Viral entry was determined using BriteLite Plus (Perkin Elmer), and the luciferase activity was measured using a Victor X3 plate reader (Perkin Elmer). Data analysis was performed using GraphPad Prism software.

**HIV-1 Env surface staining.** HEK293T cells were transfected with plasmids expressing HIV-1 Env variants lacking the cytoplasmic tail ( $\Delta$ CT) and Tat. At 48 h posttransfection, cells were detached in PBS and washed with flow cytometry buffer (PBS with 2% goat serum and 0.1% sodium azide). Cells were then incubated with increasing concentrations of 10-1074, e10-1074, CD4-mIg, 10-1074mIg, or 3BNC117mIg and incubated for 1 h on ice. To determine the binding affinities of 10-1074 and e10-1074, cells were then incubated with an allophycocyanin (APC)-conjugated anti-human secondary antibody. To determine conformational changes, cells were first incubated with CD4-mIg, 10-1074-mIg, or 3BNC117-mIg for 1 h on ice and then incubated with human 17b or mim2-Ig for 1 h on ice. Antibody binding was determined using fluorescein isothiocyanate (FITC)-conjugated anti-mouse or APC-conjugated anti-human secondary antibodies. Between each antibody incubation step, cells were washed twice with flow cytometry buffer. After incubation with secondary antibody, cells were washed once with flow cytometry buffer and once with PBS and then were resuspended in 1% paraformaldehyde in PBS. Binding was analyzed with an Accuri C6 flow cytometer (BD Bioscience). Data were analyzed with the C6 software (BD Bioscience) and GraphPad Prism.

**gp120 shedding assay.** gp120 shedding assays were performed as previously described (80, 81). To determine shedding of gp120,  $4 \times 10^5$  HEK293T cells were seeded. The following day, cells were transfected with 0.2  $\mu$ g of a plasmid expressing gp160 per well using jetPrime (Polyplus Transfection) according to the manufacturer's instructions. At 4 h posttransfection, the medium was changed. At 48 h posttransfection, medium was removed and increasing concentrations of 10-1074, e10-1074, or eCD4-Ig diluted in PBS were added to the cells and incubated for 1 h at 37°C. Supernatants were collected and centrifuged at  $1,000 \times g$  for 5 min at 4°C to remove cells and debris. Supernatants were mixed with  $2 \times$  Laemmli sample buffer containing  $\beta$ -mercaptoethanol (Sigma) and then vortexed and incubated for 5 min at 95°C. The samples were loaded onto a 4% to 12% bis-Tris gradient gel (Invitrogen) and separated by gel electrophoresis at 150 V for 80 min. Proteins were then blotted onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) using a semidry transfer cell (Bio-Rad) at 20 V for 30 min. Membranes were blocked in 5% milk for 1 h and incubated with sheep anti-gp120 polyclonal sera (NIH AIDS Reagent Program) overnight at 4°C. Membranes were then incubated with a horseradish peroxidase (HRP)-coupled rabbit anti-sheep IgG secondary antibody (Jackson ImmunoResearch), and signal was detected using a charge-coupled-device (CCD) camera (Image Quant 4000 mini; GE Healthcare).

**ADCC assay.** Antibody-dependent cell-mediated cytotoxicity (ADCC) assays were performed as previously described (75, 76). To determine ADCC activity, CEM.NKR-CCR5-LTR-Luc target cells were infected by spinoculation with 89.6 or YU2 and incubated for 3 to 4 days. On the day of the assay, infected target cells were mixed with NK effector cells at a 10:1 ratio, respectively, in the presence of increasing concentrations of 10-1074, e10-1074, 3BNC117, e3BNC117, PGT128, and ePGT128. After 8 h of incubation at 37°C, ADCC activity was determined by using BriteLite Plus, and the luciferase signal was measured using a Victor X3 plate reader. Data were analyzed using GraphPad Prism software.

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We declare the following competing interests: I.F., M.R.G., and M.F. are coinventors on a patent application describing improvements to eCD4-Ig; M.F. is an inventor on a patent describing tyrosine-sulfated coreceptor-mimetic peptides; and M.R.G. and M.F. also have financial interests in Emmune Inc., which has licensed coreceptor-mimetic peptides from Brigham and Women's Hospital and eCD4-Ig variants from the Scripps Research Institute.

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